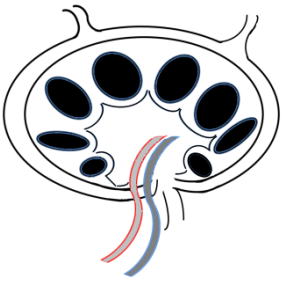
# *Chapter 7*

## Summary, discussion and future perspectives



## Summary and discussion

B cell lymphomas comprise 4% of all newly diagnosed cancer each year. Follicular lymphoma (FL) and diffuse large B cell lymphoma (DLBCL) together encompass most of the B cell lymphomas. FL is an indolent lymphoma subtype, while DLBCL is an aggressive subtype. In ten years' time approximately one-third of FL patients will transform to a more aggressive lymphoma subtype, of which DLBCL is the most common type. These lymphomas are referred to as transformed FL (TFL). FL, DLBCL and TFL belong to the non-Hodgkin lymphoma (NHL) cluster, while another essential cluster of B cell lymphoma is Hodgkin lymphoma (HL). HL is characterized by a low number of malignant B cells with an abundant background of immune cells. The aim of this PhD thesis was to investigate these lymphoma subtypes with a focus on tumor cell specific proteins and the interaction of tumor cells with reactive cells in the microenvironment.

### Proteomics studies on FL and DLBCL cell lines

In the first part of the thesis (Chapters 2 and 3), we investigated the proteomic profile of three NHL subtypes. In Chapter 2, we identified proteins differential expressed between seven NHL cell lines and an Epstein Bar virus (EBV) transformed lymphoblastoid cell line (LCL) by 2-dimensional gel electrophoresis. The intensities of 34 unique protein spots corresponding to 28 unique proteins were changed in at least three NHL cell lines in comparison to the LCL cell line. The two largest gene ontology clusters of differentially expressed proteins in our study were motility (six out of twenty eight) and metabolism (five out of twenty eight). Four of the proteins involved in motility (ARPC5, TUBB, MYL6, and CAPZA1) showed a decreased expression in NHL cell lines compared with LCLs, while expression of CFL1 and PFN1 was enhanced. Dysregulation of these proteins has been linked to metastasis and invasion in various cancer types[1,2,3]. The metabolism proteins (PKM,

IDH3, MDH1, MDH2, ENO1) were mainly upregulated in NHL cell lines compared to LCL and were involved in glycolysis and the Krebs cycle. This upregulation probably contributes to the Warburg effect that is a common anaerobic metabolic switch in cancer[4].

Expression levels of B2M, PRDX1 and PPIA were validated on the cell lines and on patient samples. Partial loss of B2M and increased levels of PRDX1 were confirmed at the mRNA level in cell lines and at the protein level in FL, TFL and DLBCL tissue samples. For PPIA a significant elevation was observed at both mRNA and protein levels in NHL compared with LCL. Treatment of NHL cells with the PPIA inhibitor Cyclosporine A (CsA) induced a dose dependent decline in the viability of malignant cells. Besides this, there was a negative correlation between PPIA expression level and the sensitivity of CsA treatment. These data suggested that upregulation of PPIA in NHLs plays a role in the malignant transformation of B cells. PPIA supports proliferation, cell cycle progression and invasion of tumors through triggering signaling pathways, such as NFAT, NF-κB, ERK1/2 and JAK/STAT[5,6]. Which of these signaling pathways is the most relevant for the CsA treatment induced anti-tumor effects needs to be further clarified.

In chapter three, we investigated the secretome of FL, germinal center B cell type (GCB) DLBCL and activated B cell type (ABC) DLBCL cell lines. In total, we identified 1,317 secreted proteins in the cell culture supernatants. Among them, 170 proteins (13%) were secreted through the classical secretion pathway and the others (n=1,147) were secreted via the non-classical pathway. Comparison of the three NHL subtypes revealed that FL and GCB-DLBCL shared more proteins as compared to the ABC-DLBCL group, consistent with the shared germinal center (GC) B cell origin. Next, we also included our previously generated secretome data of classical HL (cHL) and nodular lymphocyte predominant (NLP) HL. A small proportion of the proteins (101 proteins, i.e. 7%) were observed in the two HL and the three NHL subtypes. Both cHL (24%)

and ABC-DLBCL (18%) showed a relatively high percentage of unique proteins, whereas NLP HL had a low percentage of unique proteins similar to FL and GCB-DLBCL. The higher percentage of unique proteins in cHL might be related to the general loss of B cell phenotype previously reported in cHL[7,8].

Combination of the proteomics data with gene expression profiling data of NHL and germinal center B cells (GC-B) indicated an overlap of the secretome with differential expression patterns for 302 proteins. Most of those secreted and differentially expressed proteins (63%) were upregulated in NHLs. Gene ontology analysis of the upregulated secretome indicated that a high proportion of these proteins were involved in cell metabolism. This gene ontology was also observed as one of the main gene ontologies in chapter 2.

In summary, the two proteomics studies indicated several candidates that might be relevant for the pathogenesis of NHL and might be involved in the cross-talk between the tumor cells and the microenvironment. The overlap between the two studies is limited (upregulated ENO1, MDH2 in all NHLs; dowregulated MYL6 in DLBCLs), but this might have been caused by the differences in approach; in chapter 2 the focus was on membrane proteins and in chapter 3 we focused on secreted proteins. Nevertheless, we did see proteins related to metabolism in both studies. These findings support a role of the Warburg effect in NHL, an effect which is actually used for monitoring the tumor load in NHL patients by 2-[18F]fluoro-2-deoxyglucose positron-emission tomography (FDG-PET)[9]. Additional studies are required to further explore the potential roles of the differentially expressed secreted proteins in the cross-talk with the microenvironment and in the pathogenesis of NHLs. Moreover, it will be of interest to study if some of these proteins can be used as candidate novel biomarker for NHL diagnosis, response to treatment evaluation or as prognostic markers.

**The microenvironment of NHL**

In current studies, both reactive lymph node (RLN) and tonsil tissue samples have been used as normal controls for studies focusing on the microenvironment of NHL. As it is well known that there are several structural differences, and the reason for removal is different, we first studied these two tissue subtypes in chapter 4. We compared the difference in composition of tonsil and RLN by flow cytometry. In addition, we identified differentially expressed genes in CD4+ and CD8+ T cells.

We observed more activated T cells and Th cells, Th2 cells, and less suppressed Th cells, CTLs and NK cells in tonsils compared with RLN. In addition, we observed a lower percentage of CD127low Treg and higher percentage of GITR+ Tregs in tonsil than in RLN. Since CD127low Tregs have strong inhibitory function[10] and GITR+ Tregs convert the function of other Tregs[11], this indicates a decrease in the suppressive status of Th cells in tonsils. The tonsils have been removed because of recurrent bacterial infections, while RLN were mainly removed for differential diagnosis of lymphoma and may be more affected by (sub-) acute viral infections. Consistent with ongoing antigenic stimulation, we observed a higher frequency of activated T cells and Th2 cells in tonsils. Chronic activation of CTLs and NK cells, eventually leads to activation-induced cell death (AICD)[12]. Moreover, the increase in Th2 cells results in a decreased frequency of CTLs due to production Th2 type cytokines and lack of help to CTLs. These processes might thus explain the lower frequencies of CTLs and NK cells in tonsil compared with RLN.

At the gene expression level, no significant differences were observed between non-activated and activated CD4+ and CD8+ T cells between tonsil and RLN. This shows that despite several significant differences in the cellular composition, the functionality of the T cells is similar in both tissue types.

In summary, we found several differences between tonsil and RLN. These differences are probably related to structural differences between tonsil and RLN. Based on these findings and the common lymph node localization of lymphomas, we concluded that RLN is the preferred normal counterpart for studying the cellular composition of the microenvironment.

In chapter 5, we investigated the composition of the microenvironment of FL, TFL and DLBCL. We observed that there were less natural killer (NK) cells, macrophages in NHLs than RLN. This indicates a diminished capability to clear tumor antigens. The chronic stimulation of the immune cells by the malignant cells further leads to a suppressive phenotype of the NK cells [13,14].

To exclude the impact of variations in the percentage of tumor cells, we examined differences in the composition of Th and CTL subpopulations only in the T cell population. Differences in Treg populations were determined by flow analysis of four Treg hallmark proteins, i.e. FoxP3, GITR, CTLA4, CD127. Differences were observed only for the proportion of CD127low Tregs, which was lower in DLBCL than in RLN. These naturally occurring Tregs have a strong suppressive function[15], indicating that the immune response status in DLBCL was less suppressive compared to RLN. Notably, the proportion of TIA-1+ CTLs was elevated in NHLs compared with RLN, together this suggests that the CTLs might not function properly.

The percentage of terminal differentiated effector T cells (TEMRA) in the microenvironment of NHLs was lower than in RLNs. TEMRA cells are in the final stage of T cell differentiation and these cells retain only part of their effector function, while their proliferative potential has been lost[16]. In addition, TEMRA is a hallmark for cell senescence[17]. This indicates that the immune response is still active in NHL.

Comparison of the three different NHL subtypes to each other revealed more T follicular helper (Tfh) cells and less CD8+ CTLs, TIA-1+ CTLs

and Granzyme B+ CTLs in FL than DLBCL. The decreased percentage of Tfh cells in DLBCL is the effect or the cause of the loss of the normal germinal center structure in DLBCL. The increased proportion of CTLs in DLBCL did not prevent lymphomagenesis. This might be caused by diminished effectivity of the CTLs, or by the loss of HLA class I expression on the malignant cells. We showed functional loss of HLA class I/B2M in DLBCL cell lines and patient samples (Chapter 2), consistent with previous studies. Furthermore, a correlation has been reported between the number of CTLs and loss of HLA class I in DLBCL. This is consistent with the high expression of MIF in the secretome of NHL (Chapter 3), which can contribute to the inhibition of the cytotoxicity of CTLs.

The TFL subgroup is a heterogeneous group containing patients during transformation and patients after transformation. In the flow cytometry results we checked if this grouping could be seen, but saw no division based on the transformation status. The only differences between FL and TFL were the decreased numbers of activated CD3+ cells and CTLs in TFL. This suggests that the differences in the frequency of activated T cells were mainly caused by the decrease of activated CTLs and not by decrease of Th cells. The differences between TFL and DLBCL were increased numbers of activated T cells, activated CTLs, Granzyme B+ CTLs and decreased numbers of CD127low Tregs in DLBCL than in TFL. This might indicate that the immune response status in TFL is more suppressed than in DLBCL.

Comparison of GCB-DLBCL with ABC-DLBCL, revealed less CD57+ CD4+ cells, TIA-1+ CD4+ cells and CXCR4+ CD8+ and more CD127low Tregs in GCB-DLBCL. The higher frequency of CD57+ CD4+ cells and lower frequency of CD127low Tregs in ABC-DLBCL might also have an impact on the CD4+ cell mediated immune response. The lower frequency of TIA-1+ CD4+ cells indicates that the antigen stimulation level in GCB-DLBCL is lower than in ABC-DLBCL. Naoki et al, found

that the expression level of CXCR4 on CTLs had a negative correlation with perforin expression on CTLs[18]. This indicates that higher percentage of CXCR4+ CD8+ in ABC-DLBCL than GCB-DLBCL is associated with an enhanced suppression of the cytotoxic nature of CTLs in ABC-DLBCL.

In order to solve the questions whether the Th and CTLs cells in the microenvironment of NHLs are functionally different from those in normal lymphoid tissue we performed gene expression profiling. We activated half of the T cells by adding αCD3 and αCD28 because chronic antigen stimulation can induce T cell anergy and exhaustion[19,20]. We observed marked changes in expression level before and after stimulation, showing that the CD4+ and CD8+ can be further stimulated and are not anergic, but exhausted[19]. Exhaustion of T cells is defined as a hypo-functional status with decreased secretion of cytokines against antigens and loss of cytotoxicity[21]. In contrast, we only observed minor differences between NHL and RLN. This might in part be due to the low number of samples included in our study. Our main finding in CD8 activated T cells is increased expression of T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) in all NHLs compared to RLN. TIGIT is a co-inhibitory molecule that inhibits the cytotoxicity of CTLs. Moreover, high expression of TIGIT on T cells is a strong indication of T cell exhaustion[20], since the hallmark of exhausted T cells is elevated expression of inhibitory receptors[22]. Blocking of TIGIT can re-establish the cytotoxic function of CTLs[23]. This suggests that although we observed a high frequency of CTLs in the microenvironment of NHLs, these cells might be hypo-responsive to malignant cells and that a novel treatment, aiming to regain cytotoxic function of CTLs by adding blocking TIGIT antibody, might have potential.

To exclude bias in our flow analysis data based on the decision to use RLN as the normal control tissue, we re-analyzed the flow data using tonsil as the normal counterpart (Table 1). Five out of eleven cell

subpopulations with significant differences in the comparisons between NHL and RLN were also significantly different in the comparison between NHL and tonsil and six differences were lost. The only novel difference specific for tonsil was the increase of CD8+Granzyme B+ cells in NHL compared to tonsil. This indicates that the choice between the use of RLN and tonsil for the comparison to NHL is essential for the outcome of the study. These two normal tissue samples should not be considered as being the same when investigating the composition of the reactive infiltrate of B cell lymphomas. At the gene expression level, no difference was found between tonsils and RLN. Overall, we conclude that RLN is a more appropriate normal tissue control.

**The microenvironment of cHL**

In chapter 6, we compared the differences in the composition of the microenvironment of cHL and RLN. The frequency of GITR positive Tregs as well as FoxP3 positive Tregs were elevated in both EBV+ and EBV-cHL. This observation indicates that the tumor cells of cHL induce an immune suppressive environment irrespective of the EBV status. In a direct comparison of EBV- and EBV+ cHL, we observed more NK cells and CTLs in EBV+ cHL. This suggests that the presence of EBV antigens triggered the cytotoxic immune response. This is consistent with the much more common retention of HLA class I in EBV+ compared to EBV- cHL[24]. However, co-existence of immune cells associated with a suppressive and an activating immune response in the microenvironment of cHL, warrants further studies including the location within the tissue samples and the distance with respect to the tumor cells. To study this, we used CD26 to discriminate between cells in the close vicinity of the tumor cells, which are CD26 negative, and cells outside the tumor cell area, which are generally CD26 positive[25]. We observed more early activated Th cells and more FoxP3+ Th cells surrounding HRS cells compared to those cells located outside the tumor

cell area, independent of the EBV infection status. This indicates that the immune response against cHL was triggered successfully. The percentage of CTLs located outside the tumor area was much higher than surrounding the tumor cells. This implies that although CTLs were present, they cannot effectively attack the tumor cells. Instead, tumor cells in cHL were surrounded by Tregs, which are normally induced by Th2 cells. Although we did not see an increase of Th2 cells in chapter 6, this has been reported by our group[26] and others using a different approach[27]. Together our results indicate that Tregs play a main role in the modulation of the immune response against the tumor cells of cHL.

There are some similarities in the composition of microenvironment of cHL (chapter 6) and NHLs (chapter 5). The frequency of Tregs and CTLs in the microenvironment of both cHL and NHLs were higher than in RLN. In addition, the cytotoxic nature of the CTLs was suppressed, either by Tregs or by proteins secreted by the neoplastic cells. Thus, suppressive components in the microenvironment of both cHL and NHLs rescue the neoplastic cells from an effective anti-tumor response by the CTLs.

**Table 1. Comparison of cell composition of tonsil with NHLs and RLN with NHLs**

| Cell subpopulation | Tonsil vs lymphoma | RLN vs lymphoma |
|---|---|---|
| **CD8+** | + | + |
| **CD56+** | - | + |
| **CD68+** | - | + |
| **CD3+CD69+** | - | + |
| **CD8+CD69+** | + | + |
| **CD4+CD25+CD127low** | - | + |
| **CD8+TIA-1+** | + | + |
| **CD8+Grazyme B+** | + | - |
| **CD8+TIA-1+ in CD3+** | + | + |
| **Tfh in CD3+** | - | + |
| **TTD in CD3+** | - | + |
| **CD8+Granzyme B+ in CD3** | + | + |

+: significant difference; -: no significant difference

## Future perspective

Communications between tumor cells and immune cells can be divided into two ways: direct cell-cell contact and indirect contact by secretion of soluble factors by tumor cells to inhibit anti-tumor responses. Immunotherapy can be introduced into the treatment of B cell lymphoma to enhance the cell-cell contact between immune infiltrating cells and neoplastic cells.

It has become clear in recent years that new drugs targeting the microenvironment or influencing the cross-talk have a strong potential in the treatment of multiple cancer types. The PD-1 pathway is able to inhibit the activation of T cells through TCR pathways, block the inhibition of co-stimulators and multiple signal pathways such as PI3K/Akt, Ras-ERK1/2 in immune cells. Besides these mechanisms PD-1 is also capable to shorten the T cell-APC contact time, which will decrease the effectiveness of the immune response against neoplastic cells. Therefore, the usage of anti-PD1 drugs, Nivolumab as well as Pembrolizumab can potentially re-establish T cell and NK cell anti-tumor function[28][29]. In addition to anti-PD1 drugs, combination treatment with other immune regulators can further enhance the immune response. Examples of potentially interesting drugs are anti-CTLA4 drug, ipilimumab[30]; Bruton tyrosine kinase inhibitor, Ibrutinib[30]; T cell co-stimulator and NK cell receptor stimulator, urelummab, lirilumab[31]; Indoleamine2,3-dioxygenase inhibitor, INCB024360[30]. For CD70 (SGN-75) clinical trials are just starting and this might also be potentially interesting for NHL. TIGIT could be another potential target, but no commercial drug is available yet. The main challenge is to enhance the cytotoxic potential of CTLs and NK cells resulting in enhanced anti-tumor responses and at the same time reduce the potential of the tumor cells to secrete inhibitory molecules.

Besides cell-cell contact mediated direct communication, secreted proteins also play an essential role in the regulation of cell signaling

pathways and cell migration. Combination of the membrane proteome and secretome of NHL cell lines will allow us to predict which proteins are involved in direct and indirect cross-talk between tumor cells and their microenvironment. To fully explore our data, it is important to perform additional experiments on primary NHL tumor cells. Preferably, proteomics should be performed on sorted neoplastic cells from primary cases by for example Super-SILAC to not only identify, but also quantify the proteome of NHL tumor cells[32]. In order to generate a full interaction picture, it is necessary to also define the membrane proteins of sorted T cell subsets obtained from NHL cell suspensions. To complete this with the T cell secretome will be challenging since it is hard to get sufficient cells. As an alternative, gene expression profiling data of the CD4 and CD8 sorted T cells can be used. In addition, the proteome of other immune cell subtypes, such as the macrophages and the follicular dendritic cells, is still missing. When these data will become available a comprehensive picture of the interactions can be drawn. This will help to understand the pathogenesis of NHL and this might yield to the identification of new targets for treatment.

An example of a potential new therapeutic target is the finding of PPIA expression in NHLs. As a start PPIA expression should be studied on large cohorts of NHL patients and treatment with CsA could be further explored in mouse models. The expression level of PPIA on other B cell lymphomas is interesting to evaluate as well. Since, increased levels of PPIA plays a role in the consistent activation of NF-κB, PI3K and NF-AT pathways in other tumor cells and T cells [5,6,33] , it would be good to check which pathway is involved in B cells and NHL cells. The high responsiveness of NHL cell lines and the lack of response of lymphoblastoid cell lines to Cyclosporin A (CsA) treatment in our study supports a potential role of CsA treatment. CsA is generally applied to patients after transplantation to block a T cell response against the graft, CsA will also block an anti-tumor response by T cells and sometimes causes EBV+ post-transplantation lymphoproliferative disorders. The

treatment strategy for these tumors is to decrease the dose of CsA and add Rituximab to the treatment[34]. The risk of CsA treatment of NHL patients, is potential unfavorable effect on the anti-tumor response. Based on the exhaustion of T cells in the microenvironment of NHL (Chapter 5), it is possible that there is not really an anti-tumor response present. Maybe an additional beneficial effect of CsA will be the inhibition of T cells that provide the tumor cells with survival signals, or the blocking of immune suppressive cells as Tregs. What the overall effect of CsA in the treatment in NHL will be on the T cells, needs to be checked thoroughly before clinical use can commence.

The results of the secretome of the NHL cell lines should be further validated on NHL patient tissue and blood samples. This will indicate their potential value as disease biomarkers. Coupling with clinical data may identify which of these markers can improve prediction of patients' prognosis and treatment response, and their value to monitor disease progression during and after treatment.

Our proteomics data suggest the importance of genes involved metabolism in NHL. Thus, it might be worthwhile to perform a metabolomics study to investigate the changes in levels of metabolites as the end products of cellular processes. Metabolomics is a summary of changes in genome, transcriptome, and proteome. It can reflect changes in the tracts of cell activity, which have potential impacts on oncogenesis. Therefore, investigation of metabolomics of NHLs can give us not only a bio-signature of the diseases, but also can help the identification of novel biomarkers for diagnosis and prognosis, the responsiveness to the treatment as well as recurrence of disease.

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